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PATENT

Attorney Docket No. 016252-002110US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Graham *et al.*	Expected Examiner: P. Tung
Serial Number: Not yet assigned	Expected Art Unit: 1652
Filed: Herewith	
For: TENEBRIO ANTIFREEZE PROTEINS	COMMUNICATION UNDER 37 C.F.R. §§ 1.821-1.825 AND PRELIMINARY AMENDMENT

The Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

The instant application is a divisional application of U.S.S.N. 08/882,907, allowed on July 24, 2001. Applicants submit that the computer-readable form in the instant application is identical with the Substitute Sequence Listing filed in Application No. 08/882,907, filed June 26, 1997. In accordance with 37 C.F.R. § 1.821(e), please use the computer-readable form filed in Application No. 08/882,907 as the computer-readable form for the instant application. The information in the paper copy of the Substitute Sequence Listing enclosed herewith is identical to that which is in the computer readable form, as required under 37 C.F.R. § 1.821(f).
It is understood that the Patent and Trademark Office will make the necessary changes in application number and filing date for the computer-readable form that will be used for the instant application. Please amend the specification in adherence with 37 C.F.R. §§ 1.821-1.825 as follows. Applicants also submit herewith an amendment to the claims.

IN THE SPECIFICATION:

On page 1, following the subtitle "Cross Reference to Related Applications", please delete the sentence at line 10 and insert the following paragraph:

This application is a division of co-pending U.S. Patent Application Serial No. 08/882,907, filed June 26, 1997, the contents of which are hereby incorporated herein in their entirety.

On page 3, please delete the paragraph from line 7 to line 12 and insert the following rewritten paragraph:

This invention provides for the nucleic acid molecules that encode the proteins responsible for the thermal hysteresis in *Tenebrio* larvae. Nucleic acid sequencing predicts a thermal hysteresis protein (THP) having at least greater than one repeat of a 12 contiguous amino acid motif. This repeating motif is rich in cysteine and threonine (SEQ ID NO:1). In addition to the repeating motif, the N-terminus of the class of THP of this invention is a 16 amino acid motif (SEQ ID NO:3).

On page 5, please delete the paragraph from line 17 to line 22, and insert the following rewritten paragraph:

Figure 6 is an alignment chart of recombinant isoforms of THP (YL-1, YL-2, YL-4, YL-3 and 5-15 = SEQ ID NO:10, 12, 14, 16 and 18, respectively). The positions in which the nucleotide is conserved in all cDNA sequences (consensus = SEQ ID NO:20) are marked by an asterisk (*). The complete amino acid sequence is indicated only for YL-1 (SEQ ID NO:11). Residues of other isoforms (YL-2, YL-4, YL-3 and 5-15 = SEQ ID NO:13, 15, 17 and 19, respectively) which are identical to those found in YL-1 are indicated by a period (.). Differences are shown by boldface type where found. Gaps in both the cDNA and protein sequences are indicated by dashes (--------).

On page 6, please delete the paragraph from line 7 to line 21, and insert the following rewritten paragraph:

The primary structure of the mature THP is very unusual (Fig. 4) and is not similar to any other known sequence. The first 20 amino acids contain 6 Cys spaced at irregular intervals ($Cx_5Cx_2Cx_3Cx_2Cx_2C$; SEQ ID NO:22), and this sequence overlaps with the first of a series of 12-amino-acid repeats that continue until the end of the protein. Cys is repeated at 6-residue intervals throughout this region, which has the consensus

sequence CTxSxxCxxAxT (SEQ ID NO:1). The N-terminal Cys spacing has some elements in common with zinc-binding motifs (Klug &Schwabe, *FASEB J.* 9:597 (1995)). However, extensive dialysis against 10 mM EDTA or 10 mM phenanthroline, and the subsequent addition of 2 mM $ZnCl_2$ (or 2 mM $CaCl_2$) to chelator-free preparations incubated for 1 h at 22°C does not affect activity, suggesting that there is no role for divalent metal ions in TH activity. At least some of the Cys residues are involved in disulfide bridges because all activity is lost on incubation with 10 mM dithiothreitol at 37°C for 20 min, whereas no activity is lost under the same conditions in the absence of reducing agent. There is no effect of N-ethylmaleimide on TH activity, which suggests that if free Cys are present they can be modified without loss of activity.

On page 9, please delete the paragraph from line 20 to line 24, and insert the following rewritten paragraph:

The term "contiguous amino acid motif" refers to a repeating pattern of amino acids present in a polypeptide or protein. The amino acids in each repeat do not have to be the same but there should be a pattern common to all. For example, in the class of proteins of the present invention, the repeating amino acid motif, cys-thr-xaa-ser-xaa-xaa cys-xaa-xaa-ala-xaa-thr (SEQ ID NO:1), where xaa is any amino acid, is present.

On page 16, please delete the paragraph from line 6 to line 12, and insert the following rewritten paragraph:

There are numerous methods for isolating the DNA sequences encoding the antifreeze protein of this invention. For example, DNA may be isolated from a genomic or cDNA library using labeled oligonucleotide probes having sequences complementary to the sequences or subsequences disclosed herein (SEQ ID NO:2 or 5). Such probes can be used directly in hybridization assays to isolate DNA encoding THP isoforms. Alternatively probes can be designed for use in amplification techniques such as PCR, and DNA encoding THP may be isolated by using methods such as PCR (see *infra*).

Please delete three paragraphs from page 17, line 29 to page 18, line 23, and insert the following rewritten paragraphs:

All of the above methods can be used to prepare DNA encoding antifreeze protein. In PCR techniques, oligonucleotide primers complementary to the two borders of the DNA region to be amplified are synthesized. The polymerase chain reaction is then carried out using the two primers (see Innis). In the instant invention, because of the presence of repetitive motifs, the length of the THP subsequence encoded by the amplified product will depend on the template used. Because the N and C termini are unique (*i.e.*, different nucleotide sequences from the repetitive motif), to amplify the full-length THP encoding sequence, the primers of SEQ ID NO: 6 and 7 can be used.

PCR can be used in a variety of protocols to isolate nucleic acids encoding partial sequences of THP. In these protocols, appropriate primers and probes for amplifying DNA encoding partial sequences of THPs are generated from analysis of the DNA sequences listed herein. For example, the oligonucleotides of SEQ ID NO:6, 7, 8 and 9 can be used in a PCR protocol to amplify regions of DNA which encodes THPs. Once such regions are PCR-amplified, they can be sequenced and labeled oligonucleotide probes can be prepared from the sequence obtained. These probes can then be used to isolate DNA encoding the complete THP from DNA libraries.

SEQ ID NO:2 and 5 represent isoforms of naturally occurring *Tenebrio molitor* THP cDNA. They are not complete DNA gene sequences. However, the partial antifreeze nucleic acid sequence of SEQ ID NO:2 or 5 can be completed according to standard methods well known to those of skill in the art. A preferred approach for DNA isolation is RACE. Briefly, this technique involves using PCR to amplify a cDNA sequence using a random 5' primer and a defined 3' primer (5' RACE) or a random 3' primer and a defined 5' primer (3' RACE). The amplified sequence is then subcloned into a vector where it is then sequenced using standard techniques. The RACE method is well known to those of skill in the art and kits to perform RACE are commercially available (*e.g.*, 5' RACE System, GIBCO BRL, Grand Island, New York, USA).

On page 45, please delete the paragraph from line 7 to line 12, and insert the following

rewritten paragraph:

In a particularly preferred embodiment, nucleic acid sequences comprising the structural gene in question or upstream sequences are utilized for targeting heterologous recombination constructs. Utilizing the structural gene sequence information provided in SEQ ID NO:2 and 5 or the upstream or downstream sequence information provided in SEQ ID NO:10, 12, 14 and 16, one of skill in the art can create homologous recombination constructs with only routine experimentation.

Please delete the two paragraphs on page 48, line 22 to page 49, line 2, and insert the following rewritten paragraphs:

To isolate other isoforms of the THP of this invention, oligonucleotides for probes (SEQ ID NO:2 and 5), PCR primers (SEQ ID NO:6 and 7) and sequencing primers SEQ ID NO: 8 and 9) were designed based on the consensus sequence determined from sequencing TH positive clones (YL-1-4).

Aliquots of a *Tenebrio molitor* larval fat body λ-Zap cDNA library (see, Graham, *et al., Insect Biochem. Molec. Biol.* **26**:127 (1996)) were screened with the nucleic acid sequence of YL-1 from the 5' end to the stop codon (SEQ ID NO:21). Approximately 1 x 10^5 plaques were screened at moderate stringency following standard methodologies using the sequence listed above. Isolated positive plaques were subjected to *in vivo* excision using R408 helper phage (Stratagene) as per manufacturer's instructions. The double-stranded DNA obtained was purified and sequenced as above using the vector primers T7 and T3 as well as SEQ ID NO:8 and 9.

Please cancel the four pages containing SEQ ID NO:1 through SEQ ID NO:16 currently on file, and substitute therefor the accompanying paper copy of the Sequence Listing, pages 1 to 14.

IN THE CLAIMS:

Please cancel claims 1 to 35, and enter new claims 36 to 81 as follows:

36. An isolated or recombinantly expressed antifreeze protein, said protein comprising the following:

(i) a calculated molecular weight of between 7 and 13 kDa;

(ii) a thermal hysteresis activity of greater than about 1.5°C at a concentration of about 1 mg/mL;

(iii) the N-terminal amino acid motif set forth in SEQ ID NO:3;

(iv) specific binding to an antibody raised against an antifreeze protein selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15); and

(v) at least about 70% amino acid sequence identity to an antifreeze protein selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15).

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37. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein comprises at least one repeat of the 12 contiguous amino acid motif set forth in SEQ ID NO:1.

38. The isolated or recombinant antifreeze protein of claim 37, wherein the number of repeats of the motif is from 5 to 12.

39. The isolated or recombinant antifreeze protein of claim 36, wherein the calculated molecular weight of the antifreeze protein is between 8 and 12 kDa.

40. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein includes the subsequence of amino acids set forth in SEQ ID NO:4.

41. The isolated or recombinant antifreeze protein of claim 36, wherein the thermal hysteresis activity is greater than about 2°C at a concentration of about 1 mg/mL.

42. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15).

43. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein

is expressed by a baculovirus vector.

44. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is synthesized by a bacterial cell, a fungus cell, a plant cell, or an animal cell.

45. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is synthesized by a yeast cell.

46. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is synthesized by an animal cell.

47. The isolated or recombinant antifreeze protein of claim 36, wherein the nucleic acid encoding the antifreeze protein is synthesized by an insect cell.

48. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is derived from *Tenebrio* sp.

49. The isolated or recombinant antifreeze protein of claim 44, wherein the antifreeze protein is expressed externally from the cell.

50. An isolated or recombinantly expressed antifreeze protein, wherein said antifreeze protein is encoded by a nucleic acid which specifically hybridizes to SEQ ID NO:2 under stringent wash conditions of 0.2x SSC at 65°C for 15 minutes, and wherein said antifreeze protein has a thermal hysteresis activity greater than about 1.5°C at a concentration of about 1 mg/mL.

51. The antifreeze protein of claim 50, wherein the nucleic acid specifically hybridizes under highly stringent wash conditions of 0.15 M NaCl at 72°C for 15 minutes.

52. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein comprises at least one repeat of the 12 contiguous amino acid motif set forth in SEQ ID NO:1.

53. The isolated or recombinant antifreeze protein of claim 52, wherein the number of repeats of the motif is from 5 to 12.

54. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein has a calculated molecular weight of between 7 and 13 kDa.

55. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein includes the subsequence of amino acids set forth in SEQ ID NO:4.

56. The isolated or recombinant antifreeze protein of claim 50, wherein the thermal hysteresis activity is greater than about 2°C at a concentration of about 1 mg/mL.

57. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is expressed by a baculovirus vector.

58. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is synthesized by a bacterial cell, a fungus cell, a plant cell, or an animal cell.

59. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is synthesized by a yeast cell.

60. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is synthesized by an animal cell.

61. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is synthesized by an insect cell.

62. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is derived from *Tenebrio* sp.

63. The isolated or recombinant antifreeze protein of claim 58, wherein the antifreeze protein

is expressed externally from the cell.

64. An isolated or recombinantly expressed antifreeze protein, wherein said antifreeze protein is encoded by a nucleic acid which specifically hybridizes under stringent wash conditions of 0.2x SSC at 65°C for 15 minutes to the subsequence of SEQ ID NO:12 from nucleotides 105 to 359, and wherein said antifreeze protein lacks a signal sequence.

65. The antifreeze protein of claim 64, wherein the nucleic acid specifically hybridizes under highly stringent wash conditions of 0.15 M NaCl at 72°C for 15 minutes.

66. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein comprises at least one repeat of the 12 contiguous amino acid motif set forth in SEQ ID NO:1.

67. The isolated or recombinant antifreeze protein of claim 64, wherein the number of repeats of the motif is from 5 to 12.

68. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein has a calculated molecular weight of between 7 and 13 kDa.

69. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein includes the subsequence of amino acids set forth in SEQ ID NO:4.

70. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein has a thermal hysteresis activity of greater than about 1.5°C at a concentration of about 1 mg/mL.

71. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is expressed by a baculovirus vector.

72. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is synthesized by a bacterial cell, a fungus cell, a plant cell, or an animal cell.

73. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is synthesized by a yeast cell.

74. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is synthesized by an animal cell.

75. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is synthesized by an insect cell.

76. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is derived from *Tenebrio* sp.

77. The isolated or recombinant antifreeze protein of claim 72, wherein the antifreeze protein is expressed externally from the cell.

78. A liquid comprising a recombinant antifreeze protein, said antifreeze protein comprising the following:

(i) a calculated molecular weight of between 7 and 13 kDa;

(ii) a thermal hysteresis activity of greater than about 1.5°C at a concentration of about 1 mg/mL;

(iii) the N-terminal amino acid motif set forth in SEQ ID NO:3;

(iv) specific binding to an antibody raised against an antifreeze protein selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15); and

(v) at least about 70% amino acid sequence identity to an antifreeze protein selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15).

79. The liquid of claim 78, wherein the antifreeze protein comprises at least one repeat of the 12 contiguous amino acid motif set forth in SEQ ID NO:1.

80. The liquid of claim 78, wherein the concentration of antifreeze protein is between about

one part per billion (1 μg/L) to about one part per thousand (1 g/L).

81. The liquid of claim 78, wherein the thermal hysteresis activity is greater than about 2°C at a concentration of about 1 mg/mL.

REMARKS

Applicants are filing herewith a Sequence Listing in paper copy, in accordance with 37 C.F.R. §§ 1.821-1.825. It is confirmed that the contents of the paper copy and computer readable copy of the Sequence Listing filed in U.S.S.N. 08/882,907 are the same. The instant Amendment to the specification conforms the specification to the Sequence Listing, as was done and found acceptable in the parent application, U.S.S.N. 08/882,907. This Amendment also corrects minor inconsistencies in the specification. Applicants respectfully request entry of the Sequence Listing and Amendment. This Amendment contains no new matter.

In the parent application (U.S.S.N. 08/882,907, allowed on July 24, 2001) a Restriction/ Election Requirement grouped the claims into 5 inventions. Claims of Groups I and IV, drawn to nucleic acids encoding antifreeze proteins and transgenic organisms comprising the nucleic acids, were prosecuted and have been allowed.

In the present Amendment, Applicants are amending this divisional application by cancelling original claims 1 to 35 without prejudice, and entering new claims 36 to 81. Claims 36 to 81 are based substantially on the claims of Group II of the above-mentioned Restriction/ Election Requirement in the parent application, and are drawn to antifreeze proteins and use thereof.

Support for claims 36 to 81 can be found in the specification; for example, as follows:

Claim 36: original claim 9 (note that claim 36 now conforms to claim 1 as allowed in the parent application (U.S.S.N. 08/882,907, allowed on July 24, 2001);
Claim 37: original claim 9;
Claim 38: original claim 10;
Claim 39: original claim 2;
Claim 40: claim 4 as allowed in the parent application;

Claim 41: original claim 11;
Claim 42: original claim 12;
Claim 43: page 25, line 17;
Claim 44: original claims 15, 17, 19, 23, 24, 25, and 27;
Claim 45: original claim 25;
Claim 46: subset of claim 40;
Claim 47: page 25, lines 26 to 27;
Claim 48: page 5, lines 25 to 30;
Claim 49: claim 16 as allowed in the parent application;
Claim 50: subset of claim 40;
Claim 51: page 5, lines 25 to 30;
Claim 52: original claim 9;
Claim 53: original claim 10;
Claim 54: claim 36;
Claim 55: claim 4 as allowed in the parent application;
Claim 56: claim 36;
Claim 57: page 25, line 17;
Claim 58: original claim 11;
Claim 59: original claims 15, 17, 19, 23, 24, 25, and 27;
Claim 60: original claim 25;
Claim 61: page 25, lines 26 to 27;
Claim 62: page 5, lines 25 to 30;
Claim 63: claim 16 as allowed in the parent application;
Claim 64: claim 40 as allowed in the parent application; page 49, lines 3 to 9;
Claim 65: page 5, lines 25 to 30;
Claim 66: original claim 9;
Claim 67: original claim 10;
Claim 68: claim 36;
Claim 69: claim 4 as allowed in the parent application;
Claim 70: claim 36;
Claim 71: page 25, line 17;
Claim 72: original claim 11;
Claim 73: original claims 15, 17, 19, 23, 24, 25, and 27;

Claim 74: original claim 25;
Claim 75: page 25, lines 26 to 27;
Claim 76: page 5, lines 25 to 30;
Claim 77: claim 16 as allowed in the parent application;
Claim 78: page 5, lines 29 to 30; page 42, lines 15 to 18;
Claim 79: original claim 9;
Claim 80: page 42, lines 20 to 22; and
Claim 81: original claim 11.

Attached hereto is a "Version With Markings to Show Changes Made", in which amendments to the specification and claims can be seen. It is confirmed that no new matter has been entered by this Amendment.

In view of the instant Amendment and Sequence Listing, Applicants submit that the application is condition for allowance and respectfully request same.

Any fees that may be required in respect of this Amendment may be charged to Deposit Account No. 20-1430.

If the Examiner has any questions concerning this Amendment or the application, he is invited to telephone the undersigned at (415) 576-0200.

Respectfully submitted,

Kenneth A. Weber
Reg. No. 31,677

Date: 10/30/2001

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

On page 1, following the subtitle "Cross Reference to Related Applications", the sentence at line 10 has been deleted and the following paragraph has been inserted:

[Not applicable.] This application is a division of co-pending U.S. Patent Application Serial No. 08/882,907, filed June 26, 1997, the contents of which are hereby incorporated herein in their entirety.

On page 3, the paragraph from line 7 to line 12 has been rewritten as follows:

This invention provides for the nucleic acid molecules that encode the proteins responsible for the thermal hysteresis in *Tenebrio* larvae. Nucleic acid sequencing predicts a thermal hysteresis protein (THP) having at least greater than one repeat of a 12 contiguous amino acid motif. This repeating motif is rich in cysteine and threonine (SEQ ID NO:1). In addition to the repeating motif, the N-terminus of the class of THP of this invention is a [14] 16 amino acid motif (SEQ ID NO:3).

On page 5, the paragraph from line 17 to line 22 has been rewritten as follows:

Figure 6 is an alignment chart of recombinant isoforms of THP (YL-1, YL-2, YL-4, YL-3 and 5-15 = SEQ ID NO:10, 12, 14, 16 and 18, respectively). The positions in which the nucleotide is conserved in all cDNA sequences (consensus = SEQ ID NO:20) are marked by an asterisk (*). The complete amino acid sequence is indicated only for YL-1 (SEQ ID NO:11). Residues of other isoforms (YL-2, YL-4, YL-3 and 5-15 = SEQ ID NO:13, 15, 17 and 19, respectively) which are identical to those found in YL-1 are indicated by a period (.). Differences are shown by boldface type where found. Gaps in both the cDNA and protein sequences are indicated by dashes (--------).

On page 6, the paragraph from line 7 to line 21 has been rewritten as follows:

The primary structure of the mature THP is very unusual (Fig. 4) and is not

similar to any other known sequence. The first [21] 20 amino acids contain 6 Cys spaced at irregular intervals ($Cx_5Cx_2Cx_3Cx_2Cx_2C$; SEQ ID NO:[3] 22), and this sequence overlaps with the first of a series of 12-amino-acid repeats that continue until the end of the protein. Cys is repeated at 6-residue intervals throughout this region, which has the consensus sequence CTxSxxCxxAxT (SEQ ID NO:1). The N-terminal Cys spacing has some elements in common with zinc-binding motifs (Klug &Schwabe, *FASEB J.* **9**:597 (1995)). However, extensive dialysis against 10 mM EDTA or 10 mM phenanthroline, and the subsequent addition of 2 mM $ZnCl_2$ (or 2 mM $CaCl_2$) to chelator-free preparations incubated for 1 h at 22°C does not affect activity, suggesting that there is no role for divalent metal ions in TH activity. At least some of the Cys residues are involved in disulfide bridges because all activity is lost on incubation with 10 mM dithiothreitol at 37°C for 20 min, whereas no activity is lost under the same conditions in the absence of reducing agent. There is no effect of N-ethylmaleimide on TH activity, which suggests that if free Cys are present they can be modified without loss of activity.

On page 9, the paragraph from line 20 to line 24 has been rewritten as follows:

The term "contiguous amino acid motif" refers to a repeating pattern of amino acids present in a polypeptide or protein. The amino acids in each repeat do not have to be the same but there should be a pattern common to all. For example, in the class of proteins of the present invention, the repeating amino acid motif, cys-thr-xaa-ser-xaa-xaa cys-xaa-xaa-ala-xaa-thr (SEQ ID NO:1), where xaa is any amino acid, is present.

On page 16, the paragraph from line 6 to line 12 has been rewritten as follows:

There are numerous methods for isolating the DNA sequences encoding the antifreeze protein of this invention. For example, DNA may be isolated from a genomic or cDNA library using labeled oligonucleotide probes having sequences complementary to the sequences or subsequences disclosed herein (SEQ ID NO[s]:2 or 5). Such probes can be used directly in hybridization assays to isolate DNA encoding THP isoforms. Alternatively probes can be designed for use in amplification techniques such as PCR, and DNA encoding THP may be isolated by using methods such as PCR (see *infra*).

The three paragraphs from page 17, line 29 to page 18, line 23 have been rewritten as follows:

All of the above methods can be used to prepare DNA encoding antifreeze protein. In PCR techniques, oligonucleotide primers complementary to the two borders of the DNA region to be amplified are synthesized. The polymerase chain reaction is then carried out using the two primers (see[,] Innis). In the instant invention, because of the presence of repetitive motifs, the length of the THP subsequence encoded by the amplified product will depend on the template used. Because the N and C termini are unique (*i.e.*, different nucleotide sequences from the repetitive motif), to amplify the full-length THP encoding sequence, the primers of SEQ ID NO[s]: 6 and 7 can be used.

PCR can be used in a variety of protocols to isolate nucleic acids encoding partial sequences of THP. In these protocols, appropriate primers and probes for amplifying DNA encoding partial sequences of THPs are generated from analysis of the DNA sequences listed herein. For example, the oligonucleotides of SEQ ID NO[s]:6, 7, 8 and 9 can be used in a PCR protocol to amplify regions of DNA which encodes THPs. Once such regions are PCR-amplified, they can be sequenced and labeled oligonucleotide probes can be prepared from the sequence obtained. These probes can then be used to isolate DNA encoding the complete THP from DNA libraries.

SEQ ID NO[s]:2 and 5 represent isoforms of naturally occurring *Tenebrio molitor* THP cDNA. They are not complete DNA gene sequences. However, the partial antifreeze nucleic acid sequence of SEQ ID NO:2 or 5 can be completed according to standard methods well known to those of skill in the art. A preferred approach for DNA isolation is RACE. Briefly, this technique involves using PCR to amplify a cDNA sequence using a random 5' primer and a defined 3' primer (5' RACE) or a random 3' primer and a defined 5' primer (3' RACE). The amplified sequence is then subcloned into a vector where it is then sequenced using standard techniques. The RACE method is well known to those of skill in the art and kits to perform RACE are commercially available (*e.g.*, 5' RACE System, GIBCO BRL, Grand Island, New York, USA).

On page 45, the paragraph from line 7 to line 12 has been rewritten as follows:

In a particularly preferred embodiment, nucleic acid sequences comprising the

structural gene in question or upstream sequences are utilized for targeting heterologous recombination constructs. Utilizing the structural gene sequence information provided in SEQ ID NO[s]:2 and 5 or the upstream or downstream sequence information provided in SEQ ID NO[s]:10, 12, [13 and 14] 14 and 16, one of skill in the art can create homologous recombination constructs with only routine experimentation.

The two paragraphs on page 48, line 22 to page 49, line 2 have been rewritten as follows:

To isolate other isoforms of the THP of this invention, oligonucleotides for probes (SEQ ID NO[s]:2 and 5), PCR primers (SEQ ID NO[s]:6 and 7) and sequencing primers SEQ ID NO[s]: 8 and 9) were designed based on the consensus sequence determined from sequencing TH positive clones (YL-1-4).

Aliquots of a *Tenebrio molitor* larval fat body λ-Zap cDNA library (see, Graham, *et al., Insect Biochem. Molec. Biol.* **26**:127 (1996)) were screened with the nucleic acid sequence of YL-1 from the 5' end to the stop codon (SEQ ID NO:[16] 21). Approximately 1 x 10^5 plaques were screened at moderate stringency following standard methodologies using the sequence listed above. Isolated positive plaques were subjected to *in vivo* excision using R408 helper phage (Stratagene) as per manufacturer's instructions. The double-stranded DNA obtained was purified and sequenced as above using the vector primers T7 and T3 as well as SEQ ID NO[s]:8 and 9.

The four pages containing SEQ ID NO:1 through SEQ ID NO:16 have been cancelled in favor of the Sequence Listing being filed concurrently herewith.

IN THE CLAIMS:

Claims 1 to 35 have been cancelled, and new claims 36 to 81 entered as follows:

--36. An isolated or recombinantly expressed antifreeze protein, said protein comprising the following:

(i) a calculated molecular weight of between 7 and 13 kDa;

(ii) a thermal hysteresis activity of greater than about 1.5°C at a concentration of about 1 mg/mL;

(iii) the N-terminal amino acid motif set forth in SEQ ID NO:3;

(iv) specific binding to an antibody raised against an antifreeze protein selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15); and

(v) at least about 70% amino acid sequence identity to an antifreeze protein selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15).

37. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein comprises at least one repeat of the 12 contiguous amino acid motif set forth in SEQ ID NO:1.

38. The isolated or recombinant antifreeze protein of claim 37, wherein the number of repeats of the motif is from 5 to 12.

39. The isolated or recombinant antifreeze protein of claim 36, wherein the calculated molecular weight of the antifreeze protein is between 8 and 12 kDa.

40. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein includes the subsequence of amino acids set forth in SEQ ID NO:4.

41. The isolated or recombinant antifreeze protein of claim 36, wherein the thermal hysteresis activity is greater than about 2°C at a concentration of about 1 mg/mL.

42. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15).

43. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is expressed by a baculovirus vector.

44. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is synthesized by a bacterial cell, a fungus cell, a plant cell, or an animal cell.

45. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein

is synthesized by a yeast cell.

46. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is synthesized by an animal cell.

47. The isolated or recombinant antifreeze protein of claim 36, wherein the nucleic acid encoding the antifreeze protein is synthesized by an insect cell.

48. The isolated or recombinant antifreeze protein of claim 36, wherein the antifreeze protein is derived from *Tenebrio* sp.

49. The isolated or recombinant antifreeze protein of claim 44, wherein the antifreeze protein is expressed externally from the cell.

50. An isolated or recombinantly expressed antifreeze protein, wherein said antifreeze protein is encoded by a nucleic acid which specifically hybridizes to SEQ ID NO:2 under stringent wash conditions of 0.2x SSC at 65°C for 15 minutes, and wherein said antifreeze protein has a thermal hysteresis activity greater than about 1.5°C at a concentration of about 1 mg/mL.

51. The antifreeze protein of claim 50, wherein the nucleic acid specifically hybridizes under highly stringent wash conditions of 0.15 M NaCl at 72°C for 15 minutes.

52. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein comprises at least one repeat of the 12 contiguous amino acid motif set forth in SEQ ID NO:1.

53. The isolated or recombinant antifreeze protein of claim 52, wherein the number of repeats of the motif is from 5 to 12.

54. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein has a calculated molecular weight of between 7 and 13 kDa.

55. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein includes the subsequence of amino acids set forth in SEQ ID NO:4.

56. The isolated or recombinant antifreeze protein of claim 50, wherein the thermal hysteresis activity is greater than about 2°C at a concentration of about 1 mg/mL.

57. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is expressed by a baculovirus vector.

58. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is synthesized by a bacterial cell, a fungus cell, a plant cell, or an animal cell.

59. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is synthesized by a yeast cell.

60. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is synthesized by an animal cell.

61. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is synthesized by an insect cell.

62. The isolated or recombinant antifreeze protein of claim 50, wherein the antifreeze protein is derived from *Tenebrio* sp.

63. The isolated or recombinant antifreeze protein of claim 58, wherein the antifreeze protein is expressed externally from the cell.

64. An isolated or recombinantly expressed antifreeze protein, wherein said antifreeze protein is encoded by a nucleic acid which specifically hybridizes under stringent wash conditions of 0.2x SSC at 65°C for 15 minutes to the subsequence of SEQ ID NO:12 from nucleotides 105 to 359, and wherein said antifreeze protein lacks a signal sequence.

65. The antifreeze protein of claim 64, wherein the nucleic acid specifically hybridizes under highly stringent wash conditions of 0.15 M NaCl at 72°C for 15 minutes.

66. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein comprises at least one repeat of the 12 contiguous amino acid motif set forth in SEQ ID NO:1.

67. The isolated or recombinant antifreeze protein of claim 64, wherein the number of repeats of the motif is from 5 to 12.

68. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein has a calculated molecular weight of between 7 and 13 kDa.

69. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein includes the subsequence of amino acids set forth in SEQ ID NO:4.

70. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein has a thermal hysteresis activity of greater than about 1.5°C at a concentration of about 1 mg/mL.

71. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is expressed by a baculovirus vector.

72. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is synthesized by a bacterial cell, a fungus cell, a plant cell, or an animal cell.

73. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is synthesized by a yeast cell.

74. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is synthesized by an animal cell.

75. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein

is synthesized by an insect cell.

76. The isolated or recombinant antifreeze protein of claim 64, wherein the antifreeze protein is derived from *Tenebrio* sp.

77. The isolated or recombinant antifreeze protein of claim 72, wherein the antifreeze protein is expressed externally from the cell.

78. A liquid comprising a recombinant antifreeze protein, said antifreeze protein comprising the following:

(i) a calculated molecular weight of between 7 and 13 kDa;

(ii) a thermal hysteresis activity of greater than about 1.5°C at a concentration of about 1 mg/mL;

(iii) the N-terminal amino acid motif set forth in SEQ ID NO:3;

(iv) specific binding to an antibody raised against an antifreeze protein selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15); and

(v) at least about 70% amino acid sequence identity to an antifreeze protein selected from the group consisting of YL-1 (SEQ ID NO:11), YL-2 (SEQ ID NO:13), YL-3 (SEQ ID NO:17), and YL-4 (SEQ ID NO:15).

79. The liquid of claim 78, wherein the antifreeze protein comprises at least one repeat of the 12 contiguous amino acid motif set forth in SEQ ID NO:1.

80. The liquid of claim 78, wherein the concentration of antifreeze protein is between about one part per billion (1 µg/L) to about one part per thousand (1 g/L).

81. The liquid of claim 78, wherein the thermal hysteresis activity is greater than about 2°C at a concentration of about 1 mg/mL.--